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Antimicrobial properties of *Pseudomonas* strains producing the antibiotic mupirocin

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Abstract

Mupirocin is a polyketide antibiotic with broad antibacterial activity. It was isolated and characterized about 40 years ago from *Pseudomonas fluorescens* NCIMB 10586. To study the phylogenetic distribution of mupirocin producing strains in the genus *Pseudomonas* a large collection of *Pseudomonas* strains of worldwide origin, consisting of 117 *Pseudomonas* type strains and 461 strains isolated from different biological origins, was screened by PCR for the *mmpD* gene of the mupirocin gene cluster. Five *mmpD*$^+$ strains from different geographic and biological origin were identified. They all produced mupirocin and were strongly antagonistic against *Staphylococcus aureus*. Phylogenetic analysis showed that mupirocin production is limited to a single species.

Inactivation of mupirocin production leads to complete loss of *in vitro* antagonism against *S. aureus*, except on certain iron-reduced media where the siderophore pyoverdine is responsible for the *in vitro* antagonism of a mupirocin-negative mutant. In addition to mupirocin some of the strains produced lipopeptides of the massetolide group. These lipopeptides do not play a role in the observed *in vitro* antagonism of the mupirocin producing strains against *S. aureus*.

Key words: mupirocin; massetolide; pyoverdine, *Pseudomonas fluorescens* NCIMB 10586; *Pseudomonas* sp. W2Aug9

1. Introduction

Mupirocin is a polyketide antibiotic which has been isolated and characterized from the soil bacterium *P. fluorescens* NCIMB 10586 [1]. In fact mupirocin is a mixture of four pseudomonic acids (A-D). The basic structure of mupirocin comprises a monic acid (a heptaketide) containing a pyran ring, attached to 9-hydroxynonanoic acid via an ester linkage [2]. Mupirocin has a broad spectrum activity against both Gram-positive and Gram-negative bacteria, although most Gram-negative bacteria tested are less susceptible than Gram-positive bacteria [3]. The antibiotic acts through the inhibition of bacterial isoleucyl-tRNA synthetase [4]. Mupirocin is currently used topically for the treatment of skin infections, impetigo and for the decolonization of patients with nasal carriage of *Staphylococccus* [5].

Although the biosynthetic pathway and the antimicrobial activity of mupirocin have been extensively studied [6], the strain producing this antibiotic is not well characterized. The taxonomic position of strain *P. fluorescens* NCIMB 10586 is not known since its 16S rRNA gene nor any other housekeeping gene have been sequenced yet. It is also not known whether the ability to produce mupirocin is widespread throughout the genus *Pseudomonas* or limited to only a few species. Besides *P. fluorescens* NCIMB 10586, another two mupirocin producing strains, strain D7 and G11 isolated from groundwater sediment samples, have been reported [7]. Both strains were allocated to the *P. fluorescens* group [7] based on their 16S rRNA sequence.

The aim of this study was dual; first to assess the distribution of mupirocin producing *Pseudomonas* spp. throughout the genus *Pseudomonas* and to subsequently study their diversity. Therefore a specific mupirocin primer set was developed based on the *mmpD* gene. This multifunctional gene is involved in the synthesis of the backbone of monic acid. A large collection of *Pseudomonas* type strains and partially identified environmental strains representing a high phylogenetic diversity was screened using the mupirocin primer set.

Mupirocin production was confirmed through isolation and identification of the antibiotic and *in vitro* antagonism tests against *Staphylococcus aureus*. The second part of the work focused on the mupirocin producing strains themselves whereby it was investigated whether the strains produce additional well-known antimicrobial metabolites and extracellular enzymes to gain insight in their antimicrobial potential. The respective role of the antimicrobial compounds in the *in vitro* antagonism against *S. aureus* was further investigated via transposon mutagenesis.

2. Material and Methods

2.1. Bacterial strains

A total of 578 strains and isolates from different biological origins were studied. These included 117 type strains of the genus *Pseudomonas* (listed in Supplementary Table S1) and a collection of 198 *Pseudomonas* isolates from the River Woluwe, which were isolated from the source (site W2) and the mouth of the river (site W15) (Supplementary Table S2 in [8]). In addition, a collection of 48 *Pseudomonas* non type strains obtained from different culture collections (Supplementary Table S2) were included together with 215 strains and isolates received from different laboratories (Supplementary Table S3).

2.2. Growth conditions

The *Pseudomonas* strains (Table 1) were routinely grown at 28°C on an in house medium, medium 853, composed of 10 g l^{-1} bacto tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 1 g l^{-1} glucose, 0.7 g l^{-1} K_2HPO_4 and 0.3 g l^{-1} KH_2PO_4. *Escherichia coli* SM10 (λpir) was grown in 853 at 37°C. Gentamicin (Gm) was used at a concentration of 50 µg ml^{-1} (*P. fluorescens* NCIMB 10586) or 200 µg ml^{-1} (*Pseudomonas* sp. W2Aug9), chloramphenicol (Cm) was used at a concentration of 25 µg ml^{-1}. Antagonism tests were performed on 4 different media;

medium 853, blood agar (BBL^{TM} Blood agar Base [Becton, Dickinson and company] with 5 % of defibrinated sheep blood), Mueller-Hinton agar (MH) (Difco Laboratories) and GCA medium [9]. When required GCA was supplemented with $FeCl_3$ to a final concentration of 100 µM (GCA+Fe medium). Pyoverdine detection and purification was done on iron-poor casamino acids (CAA) medium [10].

2.3. Screening for mupirocin producing strains by PCR

During a PCR screening of 195 *Pseudomonas* strains for the antibiotic pyoluteorin with the primer set PltBf and PltBr [11] two false positives were picked up of which the nucleotide sequence aligned with part of the *mmpD* gene sequence (position 21309-22090) of the mupirocin gene cluster of *P. fluorescens* NCIMB 10586 (accession number AF318063). Comparing the sequence of the PltBf-r primer set with the complementary sequence of the *mmpD* gene of *P. fluorescens* NCIMB 10586 showed that there was only 1 mismatch with the PltBf primer and several for the PltBr primer (Supplementary Fig. S1). The combination of one almost perfect primer with the relatively low annealing temperature during the amplification can explain why *mmpD* fragments were obtained during the screening with the pyoluteorin primer set. A specific mupirocin primer set (MUP-F1: 5'-CGGATCATGGACCCCCAGC-3' and MUP-R1: 5'-CAGGCCTTGGATCTCGATAG-3') was developed on the basis of the PltBf primer and the *mmpD* sequences of *P. fluorescens* NCIMB 10586 and the 2 candidates obtained through the screening with the pyoluteorin primer set. They amplify a fragment of 717 bp. All 578 strains and isolates were screened with MUP-F1 and MUP-R1. The PCR was carried out in a final volume of 25 µl containing PCR buffer (Qiagen) with 0.625 U *Taq* DNA polymerase (Qiagen), 5 µl Q-solution (Qiagen), the deoxynucleotide mixture at 100 µM (Fermentas), each of the primers at 0.5 µM (Sigma) and 1-2 µl template DNA. Template DNA for PCR reaction was prepared by inoculating a

fresh colony in 25 µl water and heating it for 5 min at 95°C. The PCR program used was an initial denaturation of 2 min at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 50 s, followed by an incubation for 10 min at 72°C. *P. fluorescens* NCIMB 10586 was used as positive control. The obtained PCR fragments were sequenced directly using the amplification primers at Beckman Coulter Genomics (UK). The accession numbers of the *mmpD* fragment of *P. fluorescens* LMG 14677, *Pseudomonas* sp. W2Aug9, W2Jun17, W15Feb34 and B329 are KJ528554, KJ528555, KJ528556, KJ528557 and KJ528558, respectively.

2.4. Mupirocin production

Mupirocin was purified from the culture supernatant as described in [12]. The presence of the antibiotic was determined by HPLC with a reverse-phased C18 Altima (GRACE) column (250 mm x 4.6 mm, 10 µM), using the conditions as described in [12]. Mupirocin was detected at 27.6 min. Pure mupirocin (Sigma) was used as control.

2.5 PCR for the tailoring enzymes of the mupirocin gene cluster

To detect the genes responsible for the maturation of pseudomonic acid A in the five mupirocin producing strains 26 primer sets (Supplementary Table S4) were developed to amplify part of these genes. PCR was done with DreamTaq Green (Thermo Scientific) using the same conditions as for the mupirocin PCR. *P. fluorescens* NCIMB 10586 was used as positive control. For primer set mupU-F/R the PCR was done at 59°C since lower temperatures gave an amplicon of lower size for *Pseudomonas* sp. W2Aug9, W2Jun17 and *P. fluorescens* LMG 14677.

2.6. 16S rRNA and rpoB *gene amplification and sequencing*

The almost complete 16S rRNA gene sequence (position 29 to 1522 in *E. coli*) of the *mmpD*$^{+}$ *Pseudomonas* strains was amplified with primers pA and pH [13]. The PCR mix was prepared using the same conditions as described for the mupirocin PCR with the difference that the PCR was carried out in a final volume of 100 µl and the amount of template was increased to 3 µl. The PCR program used was an initial denaturation of 2 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 s, followed by an incubation for 10 min at 72°C. The accession numbers of *P. fluorescens* NCIMB 10586, *P. fluorescens* LMG 14677, *Pseudomonas* sp. W2Aug9, *Pseudomonas* sp. W2Jun17, *Pseudomonas* sp. W15Feb34 and *Pseudomonas* sp. B329 are KJ528545, GU198127, KJ528546, KJ528547, EU681017, KJ528548, respectively. The housekeeping gene *rpoB* was amplified using primers LAPS and LAPS27 [14] using the same conditions as for the mupirocin PCR, except that the elongation time was increased to 1 min 20 s. The obtained PCR fragments were purified and sequenced at Beckman Coulter Genomics (UK) using amplification and internal primers (for 16S rRNA).

2.7. Phylogenetic analysis

Phylogenetic analysis based on almost complete 16S rRNA gene sequences and partial *rpoB* genes were performed using CLUSTALX and MEGA v5.0 [15]. The neighbor-joining method was used with the Jukes-Cantor model and topological robustness was evaluated by bootstrap analysis based on 1.000 replicates. The 16S rRNA gene sequences of the type strains and the *rpoB* gene of *P. aeruginosa* LMG 1242$^{\mathbf{T}}$ were taken from GenBank.

2.8. Antibiotic and HCN production

Detection of the genes responsible for the production of hydrogen cyanide (HCN) [16] and antibiotics was done by PCR using gene-specific primers. The antibiotics of interest are

2,4-diacetylphloroglucinol [17], pyrrolnitrin [17] and phenazine-1-carboxylic acid [18]. The same conditions as for the mupirocin PCR were used except that the elongation time was adapted to the size of the fragment. *P. protegens* Pf-5 was used as positive control.

2.9. Protease production

Protease activity was detected as clearing zones on skim milk agar plates [19].

2.10. Pyoverdine production

Pyoverdine production was verified by measuring the OD at 405nm of the culture supernatant of the cells grown in CAA medium for 48 h at 28°C, 200 RPM. The pyoverdine was semi-purified as described in [20] and identified through a combination of mass analysis and IEF [20].

2.11. Production and identification of lipopeptides

Surfactant production was verified through the drop collapse assay. Therefore bacterial cells were grown for 24h and 10 µl droplets of the culture or the culture supernatant were spotted on parafilm. A flat droplet was indicative of lipopeptide production. Hemolytic activity was detected as a lysis zone surrounding colonies grown for 3 days at 28°C on blood agar.

Lipopeptides were analyzed from supernatant samples with a UPLC (Acquity H-class, Waters s.a., Zellik, Belgium) coupled to a single quadrupole mass spectrometer (Waters SQD mass analyzer) on a ACQUITY UPLC® BEH C_{18} 1.7 µm column. Ten µl was injected and elution was performed at 40°C with a constant flow rate of 0.6 ml min^{-1} using a gradient of acetonitrile in water both acidified with 0.1% formic acid as follows: 0.5 min at 0%, from 0% to 15% in 2 min, from 15% to 95% in 5 min and maintained at 95% for 1.8 min. Compounds

were detected as protonated molecular ions detected in electrospray positive ion mode (scan in the mass range *m/z* 950-1250) by setting SQD parameters as follows: source temperature 130°C; desolvation temperature 300°C, and desolvation/cone nitrogen flow: 1000/50 l h^{-1}. For optimal detection, the cone voltage was set at 80V. The same conditions were used for analysis of lipopeptide in source-fragmentation but the cone voltage was increased to 120V.

2.12. In vitro antagonism assays against S. aureus

The *in vitro* antagonism of the mupirocin producing *Pseudomonas* strains against *S. aureus* 383, a clinical strain isolated from infected femoral pin [21] was tested on 853 agar, blood agar, MH agar, GCA and GCA amended with 100 µM Fe. Five µl of OD_{600} = 0.5 of the *Pseudomonas* strain was inoculated in the center of the plate. The plates were incubated at 28°C and after 2 days the cells were killed using chloroform vapors for 25 min. After evaporation of the chloroform a 6 ml overlay of soft agar (0.7% agar) of 853 with 5 x 10^6 cells of *S. aureus* 383, was overlaid on the plate which was subsequently incubated at 37°C. Clear zones of inhibition were measured the next day. All antagonism assays were performed at least two times with 3 replicas.

2.13. Transposon mutagenesis and screening for a hemolytic-negative mutant of Pseudomonas *sp. W2Aug9*

The suicide plasmid pUT, which harbors the transposon mini-Tn*5phoA3* (Gm^R) [22] was used to generate transposon insertions in the chromosome of *Pseudomonas* sp. W2Aug9. Mid-log phase cultures of *E. coli* SM10 (λpir), the host of pUT-mini-Tn*5phoA3*, was mixed with strain W2Aug9 in a 1:1 ratio. The *Pseudomonas* strain was kept at 37°C for 1 h just before mixing with *E. coli* in order to inactivate its restriction system. After overnight incubation on 853 at 28°C, Tn*5* insertions were selected on CAA supplemented with 25 µg

216 ml^{-1} Cm and 200 µg ml^{-1} Gm. A small bank of 468 transconjugants was established to screen
217 for mutants showing complete loss of hemolytic activity by inoculating with a toothpick
218 mutants on a blood agar plate followed by incubation for 2 days at 28°C. Hemolytic-negative
219 candidates were subsequently verified for protease production.
220
221 *2.14. Transposon mutagenesis and screening for an antagonism-negative mutant of*
222 10586ΔAT2
223 An antagonism-negative mutant of the mupirocin-negative strain 10586ΔAT2 of *P.*
224 *fluorescens* NCIMB 10586 was obtained through Tn*5* mutagenesis on 10586ΔAT2 using
225 mini-Tn*5phoA3* as described in 2.13, with the modification that the transconjugants were
226 plated on a lower Gm concentration (50 µg ml^{-1}). A library of 2000 candidates was screened
227 for complete loss of *in vitro* antagonism against *S. aureus* 383. Therefore the candidates were
228 grown overnight at 28°C in medium 853 in microtiter plates whereby every two wells were
229 inoculated, alternating between an inoculated and not inoculated well. The cultures were
230 replicated on 853 medium and incubated for 16h at 28°C. An overlay of *S. aureus* 383 was
231 prepared as explained in paragraph 2.12.
232
233 *2.15. Transposon mutagenesis and screening for a pyoverdine-negative mutant of* P.
234 fluorescens *NCIMB 10586*
235 A pyoverdine-negative mutant of *P. fluorescens* sp. NCIMB 10586 was obtained
236 through Tn*5* mutagenesis using mini-Tn*5phoA3* as described in 2.13. Tn*5* insertions were
237 selected on CAA supplemented with 50 µg ml^{-1} Gm and 25 µg ml^{-1} Cm. A bank of 576
238 transconjugants was screened for complete loss of fluorescence in the iron-limiting media
239 CAA.
240

*2.16. Molecular characterization of the Tn*5 *mutants*

The chromosomal DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen), digested with *Pst*I or *Sal*I (Fermentas) and self-ligated. The DNA flanking the mini-Tn*5phoA3* was isolated and sequenced as described earlier [23].

3. Results

3.1. Screening for mupirocin producing strains

578 *Pseudomonas* strains were screened by PCR for presence of part of the *mmpD* gene fragment. Five strains (0.9% of the collection) gave an amplicon. Three of these strains (W2Aug9, W2Jun17 and W15Feb34) were from the Woluwe River collection [8]. In addition the *mmpD* gene fragment was amplified from *P. fluorescens* LMG 14677 and *Pseudomonas* sp. B329, a grass rhizosphere isolate. Sequencing of the *mmpD* fragments of the 5 candidates and alignment with the gene of *P. fluorescens* NCIMB 10586 revealed that *Pseudomonas* sp. W15Feb34 and B329 have identical sequences to NCIMB 10586, W2Aug9 has a C→T transition at position 255 of the amplified fragment and *P. fluorescens* LMG 14677, W2Jun17 and W2Aug9 an A→G transversion at position 489. These differences do not alter the amino acid sequence.

Purification of mupirocin from these strains and analysis by HPLC confirmed that they all produce the antibiotic. Mass analysis of the obtained peaks identified pseudomonic acid A and B for all the strains (Supplementary Fig. S2). In contrast to what has been reported previously [6] whereby pseudomonic acid B represented only a minor fraction of the mixture (8%), higher levels of pseudomonic acid B were found (relative abundance between 29 and 61%) in all producing strains including *P. fluorescens* NCIMB 10586. This is probably due to differences in growth conditions and/or media. PCR with primers specific for the tailoring genes of the mupirocin gene cluster (results not shown) showed that all the strains share the

same genes for the maturation of pseudomonic acid A, including the quorum sensing/quenching system encoded in the gene cluster.

The amount of mupirocin produced in the culture supernatant was between 1-15 µg/ml depending of the producing strain; *P. fluorescens* LMG 14677 and W15Feb34 produced similar amounts, 6.7 and 5.8 µg/ml respectively, as *P. fluorescens* NCIMB 10586 (8.3 µg/ml). B329 produced about half of the amount (4.5 µg/ml) of NCIMB 10586. Relatively low concentrations were detected for W2Jun17 which produced only about 1/10 of the amount observed for NCIMB 10586 (1.0 µg/ml). W2Aug9 showed the highest level of mupirocin production, producing twice as much as NCIMB 10586 (15.2 µg/ml). These values are in the same range as those reported for strains D7 and G11 (1.8-3.5 µg/ml) [7]. The differences in mupirocin production between the strains were not reflected in their degree of *in vitro* antagonism against *S. aureus* which were similar. The antagonism of NCIMB 10586 and W2Jun17 showed the lowest level (24.8 ± 0.5 mm and 24.4 ± 0.5 mm, respectively), followed by LMG 14677 (25.7 ± 1.2 mm), W15Feb34 (26.7 ± 1.0 mm) and W2Aug9 (27.3 ± 1.3 mm). The largest inhibition zone was observed for B329 (29.0 ± 0.5 mm). The strains showing the lowest level of antagonism were the strains that do not produce lipopeptides of the massetolide group, these results indicate that production of massetolide might slightly increase *in vitro* antagonism.

3.2. Identification of mupirocin producing strains

Alignment of the 16S rRNA sequences showed that all the mupirocin producing strains had identical sequences. Inclusion of the 16S rRNA genes of strain D7 and G11 [7] in the alignment showed that both strains had also the same 16S rRNA sequence (after truncating the 5’ and 3’-end by maximum 14 nucleotides to remove evident sequencing errors) showing that in fact all mupirocin producing strains belong to the same species.

To identify the strains a phylogenetic tree was constructed based on the 16S rRNA nucleotide sequence of the selected representative strain *P. fluorescens* NCIMB 10586 and a selection of *Pseudomonas* type strains from the *P. fluorescens* group. *P. fluorescens* NCIMB 10586 belongs to the *P. fluorescens* group and the closest type strain to strain NCIMB 10586 is *P. azotoformans* IAM 1603 with a bootstrap value of 53% (Fig. 1.A.). The closest relatives were *P. cedrina* subsp. *cedrina* CFML 96-198^{T}, *P. cedrina* subsp. *fulgida* DSM 14938^{T}, *P. libanensis* CIP 105460^{T} and *P. synxantha* IAM 12356^{T} (Fig. 1.A.).

The intraspecific diversity of the mupirocin producing strains was assessed on the basis of the sequence of the housekeeping gene *rpoB*. A tree was constructed using the same methodology as for 16S rRNA. The strains formed a clear separate cluster and a unique *rpoB* sequence was found for each strain (Fig. 1.B.).

3.3. Production of antimicrobial metabolites and protease

The six mupirocin producing strains were screened for additional well-known antimicrobial metabolites and protease. They were screened by PCR for HCN and the antibiotics 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin and phenazine-1-carboxylic acid. In the conditions used no amplification was obtained for any of these compounds. All the mupirocin strains produced protease and had similar clear zones around the colony. They all produced the siderophore pyoverdine. Pyoverdines are made of three distinct structural parts: a small peptide chain of 6 to 14 L- and D-amino acids, linked to a yellow-green chromophore group and to a small dicarboxylic acid [24]. Mass analysis showed that all strains produced the same pyoverdine PYO_{13525}, with a peptide chain of 7 amino acids with sequence Ser-Lys-Gly-FOHOrn-(Lys-FOHOrn-Ser), the parentheses indicate a cyclic structure [25]. This pyoverdine is relatively common and is produced by many strains of the *P. fluorescens* group (Matthijs S., *unpublished results*).

316

317 *3.4. Several mupirocin producing strains produce lipopeptides of the massetolide group*

318 The positive drop collapse test observed for three of the six strains, namely
319 *Pseudomonas* sp. W2Aug9, *Pseudomonas* sp. B329, and *Pseudomonas* sp. W15Feb34
320 suggested that those isolates may produce compounds with surface tension reducing activity.
321 In addition, together with *P. fluorescens* LMG 14677, they displayed hemolytic activity when
322 tested on red sheep blood cells. Strain W2Aug9 had the largest activity followed by
323 *Pseudomonas* sp. B329, *P. fluorescens* LMG 14677 and *Pseudomonas* sp. W15Feb34. Those
324 phenotypes prompted us to check for possible production of surfactants by these strains.
325 UPLC-MS analysis of culture supernatants revealed that strains W2Aug9, B329, LMG 14677
326 and W15Feb34 but not NCIMB 10586 and W2Jun17 secreted cyclic lipopeptides with major
327 peaks observed at *m/z* 1126.7, 1140.7, 1154.7, 1168.7 for molecular ions (Fig. 2,
328 Supplementary Fig. S3). These molecular ions correspond to the massetolide group of
329 *Pseudomonas* lipopeptides and similar masses and retention times were observed by injecting
330 purified massetolides from *P. fluorescens* SS101 [26, 27, 28]. Further analysis of product ions
331 yielded upon in-source fragmentation of those molecular species confirmed that they
332 correspond to massetolides A/D, L and F/viscosin with either C_{10} or C_{12} fatty acid moieties.
333 Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the
334 fatty acid. The occurrence of several derivatives of the lipopeptide reflects some flexibility in
335 amino acid selection and activation by the A domains of the massetolide synthetases.

336 When comparing the level of *in vitro* antagonism of the mupirocin producing strains
337 on the different media against *S. aureus* it was observed that strains producing lipopeptides
338 generally showed a weak, but reproducible, larger zone of inhibition in the antagonism against
339 *S. aureus* then strains not producing lipopeptides. To verify whether there was a synergistic
340 effect a hemolytic-negative mutant was created in strain W2Aug9 which showed the largest

hemolytic activity. Therefore a Tn*5* bank of W2Aug9 was screened for complete loss of hemolytic activity on blood agar. In total 14 hemolytic-negative candidates were obtained. All the candidates were negative in a drop collapse test showing loss of surfactant production. Mutant W2Aug9-F1 was selected from these mutants because it was the only strain that produced similar clear zones on skim milk plates as the wild type. Sequencing and BlastX analysis of the Tn*5* flanking region of mutant W2Aug9-F1 showed that it had an insertion into a sequence which showed the highest similarity to *massA* of *Pseudomonas fluorescens* SS101 (92% identity at aa level of a 633 bp fragment). The non-ribosomal peptide synthetase enzyme MassA is responsible for the biosynthesis of the first 2 amino acids of the cyclic lipopeptide massetolide [27]. In strain *P. fluorescens* SS101 the *massA* gene is organized in a separate cluster from *massB* and *massC*.

When tested for *in vitro* antagonism against *S. aureus* the massetolide-negative mutant showed a similar level of antagonism as the wild type *Pseudomonas* sp. W2Aug9 on all media tested (results not shown) therefore ruling out a synergistic role for the lipopeptides in the *in vitro* antagonism against *Staphylococcus*.

3.5. Loss of mupirocin results in pyoverdine-dependent in vitro *antagonism*

The mupirocin producing strains were strongly antagonistic against *S. aureus* 383 on the rich media tested (853, MH and blood agar). The level of *in vitro* antagonism of the six strains was comparable (data not shown) and results are detailed only for *P. fluorescens* NCIMB 10586 (Fig. 3 and Fig. 4A-D-G). The largest inhibition zone (radius of the zone) for NCIMB 10586 was observed on 853 (23.3 ± 1.3 mm) (Fig. 4A), followed by MH (20.2 ± 1.5 mm) (Fig. 4D) and blood agar (16.3 ± 0.5 mm) (Fig. 4G). The mupirocin-negative mutant 10586ΔAT2 (Fig. 3), completely lost its ability to inhibit *S. aureus* on blood agar (Fig. 4H).

On 853 and MH medium the antagonism was strongly reduced (7.5 ± 1.3 mm) and (5.2 ± 0.8 mm), respectively (Fig. 4E and B).

To identify the nature of the compound responsible for the *in vitro* antagonism of the mupirocin-negative mutant 10586ΔAT2 on 853 (Fig. 4B) a Tn*5* mutagenesis was carried out and the library was screened for transconjugants that completely had lost the antagonism. Several mutants were obtained and molecular characterization showed that in all these mutants the Tn*5* insertions were in the NRPS genes of pyoverdine. As expected, pyoverdine was no longer produced in the mutants, strongly suggesting that pyoverdine is responsible for the antagonism of the mupirocin-negative mutant on medium 853. Mutant 10586ΔAT2-10H10 was selected, this mutant had a Tn*5* insertion in the middle of the *pvdI* gene and the translated sequence of the flanking region of the transposon showed the highest similarity to PvdI of *P. synxantha* BG33R (98% identity at aa level of a 800 bp fragment). The *in vitro* antagonism of this mutant was tested on 853, MH and blood agar (Fig. 3). On blood agar the antagonism was completely lost (Fig. 4I), on 853 (Fig. 4C) and MH agar (Fig. 4F) an inhibition zone was still observed but it was almost completely covered with *S. aureus*. Since pyoverdine was responsible for the observed *in vitro* antagonism the antagonism was also tested on the low iron media GCA and GCA amended with excess iron. On GCA and GCA+Fe medium the wild type showed a clear inhibition zone (Fig. 4J and 4M). The mupirocin-negative mutant showed a clear inhibition zone on GCA (Fig. 4K), this antagonism was the largest observed on all the media tested for this mutant (Fig. 3). With the addition of iron the antagonism was completely lost confirming that an iron regulated compound is being produced (Fig. 4N). The double mupirocin/pyoverdine-negative mutant 10586ΔdAT2-10H10 (Fig. 3) showed an inhibition zone into which *S. aureus* was growing (Fig. 4L) on GCA medium, as for the mupirocin-negative mutant the antagonism was completely lost in the presence of iron (Fig. 4O).

To look at the effect of the loss of only pyoverdine on the antagonism against *S. aureus*, a pyoverdine-negative mutant, 10586-3H3, was obtained through Tn*5* mutagenesis of the wild type NCIMB 10586. This mutant had an insertion into a gene of which the translated sequence had the highest similarity to PvdD of *P. synxantha* BG33R (98% identity at aa level of a 748 bp fragment). The *pvdD* mutant was not affected in the *in vitro* antagonism against *S. aureus* on any of the media tested (Fig. 3). The growth inhibition effect due to pyoverdine appeared to be masked by mupirocin for this strain.

4. Conclusion

The polyketide mupirocin is a valuable antibiotic, with a current therapeutic use. Until now it has only been produced from *P. fluorescens* NCIMB 10586 where its synthesis is encoded by a 65-kb long region comprising 35 genes [12, 29]. In this work, five new mupirocin producing strains were identified during the screening of a phylogenetically diverse collection of 578 *Pseudomonas* strains and isolates. Together with the two reported mupirocin producing strains D7 and G11 [7], they all belong to the same species as *P. fluorescens* NCIMB 10586. Accordingly, mupirocin production seems to be restricted to a limited number of isolates belonging to one single species which is different from the currently published *Pseudomonas* type strains.

One explanation for the apparent unique phylogenetic affiliation of the mupirocin producing strains would result from the methodology used to detect mupirocin producing isolates. Indeed, when screening for a specific gene by PCR, candidates may be missed because one or both primers do not anneal due to sequence divergence at the primer annealing site. Yet, the mupirocin producing strains D7 and G11 were isolated on the basis of the antimicrobial effect of their supernatant on Gram-positive bacteria and not by PCR. The fact

that mupirocin producing strains isolated using 2 different approaches all belong to the same species supports that mupirocin is probably produced by a restricted group of strains.

The capacity to produce mupirocin could well have been acquired by horizontal gene transfer in a recent ancestor of the species. Indeed, analysis of the flanking region of the mupirocin gene cluster suggests that the cluster may be an insertion integrated by recombination at tRNA genes as observed for a number of phage and mobile elements [12]. Interestingly, the antibiotic thiomarinol produced by the marine bacterium *Pseudoalteromonas* sp. SANK 73390 is a hybrid of two components: pseudomonic acid and pyrrothine [30; 31]. Both compounds are encoded by 2 different clusters on plasmid pTML1 [30].

Mupirocin has a broad spectrum activity and the acquisition of the mupirocin gene cluster may significantly increase fitness and competitiveness of the species. Yet as a consequence of the small number of mupirocin producing strains some uncertainty remains with respect to the true ecology of the species and its way of life.

In our collection and in the study of [7], mupirocin producing strains were predominantly isolated from water and soil environments. Only one strain, *Pseudomonas* sp. B329, was isolated from the rhizosphere of grass. Interestingly, this was the only strain of the six mupirocin producing strains which showed motility under microscope (*results not shown*). Yet, rhizosphere strains are underrepresented in the collection screened; this could explain why only one rhizosphere strain was picked up during the screening.

Given the strong antimicrobial activity of mupirocin it would be interesting to test these strains as biocontrol agents against bacterial disease. The ability of some of the mupirocin producing strains to produce lipopeptides of the massetolide group, which have surfactant and antimicrobial properties, could potentially even make the strains active against fungal diseases [32].

Acknowledgement

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Table 1. List of strains used in this study.

Strain	Relevant genotype/characteristics	Reference
Pseudomonas fluorescens NCIMB 10586	Mupirocin producing strain isolated from soil, Hampstead Heath, London, U.K.	[1]
10586ΔAT2	Mupirocin-negative deletion (MmpCΔ337-548) mutant of *P. fluorescens* NCIMB 10586	[12]
10586-3H3	Pyoverdine-negative Tn*5* mutant of *P. fluorescens* NCIMB 10586 with a Tn*5* insertion into the *pvdD* gene, Gm^R	This study
10586ΔAT2-10H10	Pyoverdine-negative mutant of 10586ΔAT2 with a Tn*5* insertion into the *pvdI* gene, Gm^R	This study
Pseudomonas fluorescens LMG 14677	Mupirocin producing strain	[33]
Pseudomonas sp. W2Aug9	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
W2Aug9-F1	Massetolide-negative Tn*5* mutant of *Pseudomonas* sp. W2Aug9 with a Tn*5* insertion into *massA*, Gm^R	This study
Pseudomonas sp. B329	Mupirocin producing strain isolated from rhizosphere of grass, France	Bodilis J., France
Pseudomonas sp. W2Jun17	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
Pseudomonas sp. W15Feb34	Mupirocin producing strain isolated from river water, Brussels, Belgium	[8]
Staphylococcus aureus 383	Clinical strain isolated from infected femoral pin, mupirocin-sensitive	[21]

Fig. 1.A. Phylogenetic relationships among 21 *Pseudomonas* type strains of the *P. fluorescens* group and *P. fluorescens* NCIMB 10586 based on 16S rRNA sequences (the accession number of the 16S rRNA sequences are indicated after the strain name). *P. aeruginosa* LMG 1242^{T} was used as outgroup to root the tree. The tree was constructed using the NJ method. Number at nodes represents levels (%) of bootstrap support from 1000 resampled datasets. The bar represents 0.01 substitutions per nucleotide position.

Fig. 1.A.

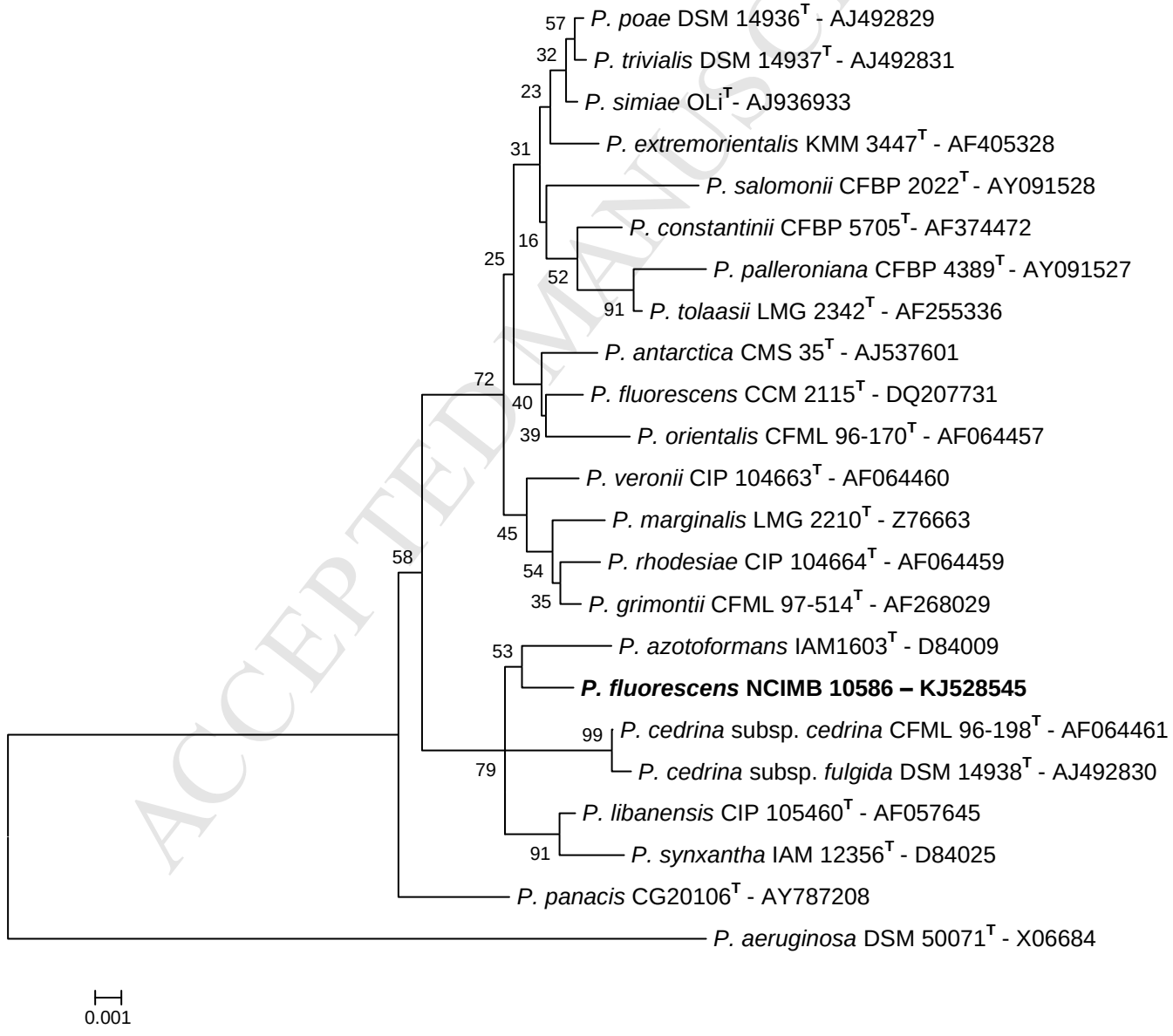


Fig. 1.B. Phylogenetic relationships among the mupirocin producing strains based on *rpoB* gene sequences (the accession number of the *rpoB* gene sequences are indicated after the strain name). *P. aeruginosa* LMG 1242^{T} was used as outgroup to root the tree. The strains with hemolytic activity are indicated by a black dot. The tree was constructed using the NJ method. Number at nodes represents levels (%) of bootstrap support from 1000 resampled datasets. The bar represents 0.01 substitutions per nucleotide position.

Fig. 1.B.

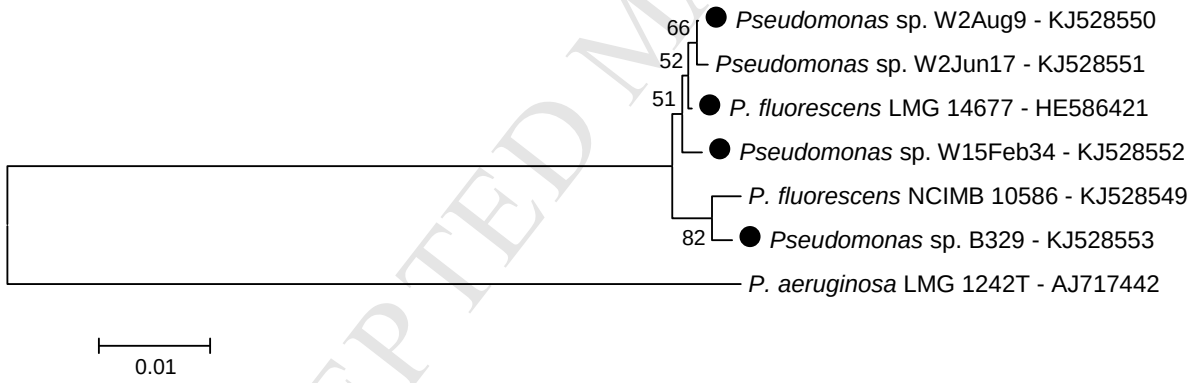


Fig 2. UPLC-MS analysis of culture supernatants of *Pseudomonas* sp. W2Aug9. Strain W2Aug9 secretes cyclic lipopeptides with major peaks observed at *m/z* 1126.7, 1140.7, 1154.7 and 1168.7 for molecular ions. Further analysis of product ions yielded upon in-source fragmentation of those molecular species confirmed that they correspond to massetolides A/D, L and F/viscosin with either C_{10} or C_{12} fatty acid moieties. Minor peaks observed at 1152.7 and 1166.7 correspond probably to one unsaturation in the fatty acid.

Fig. 2.

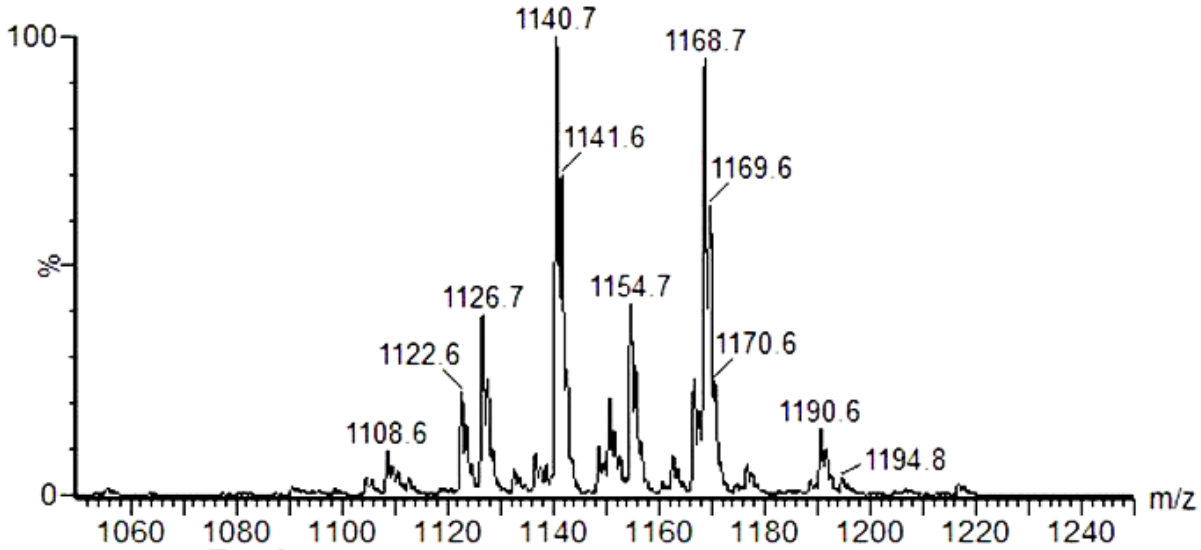


Fig. 3. The *in vitro* antagonism of the wild type *P. fluorescens* NCIMB 10586, the mupirocin-negative mutant 10586ΔAT2, the pyoverdine-negative mutant 10586-3H3 and the double mupirocin/pyoverdine-negative mutant 10586ΔAT2-10H10. The bars represent the inhibition zone (mm) observed against *S. aureus* 383. For the double mutant the color of the bar is presented as a gradient since inhibition zones were observed which were partially covered with bacteria.

Fig. 3.

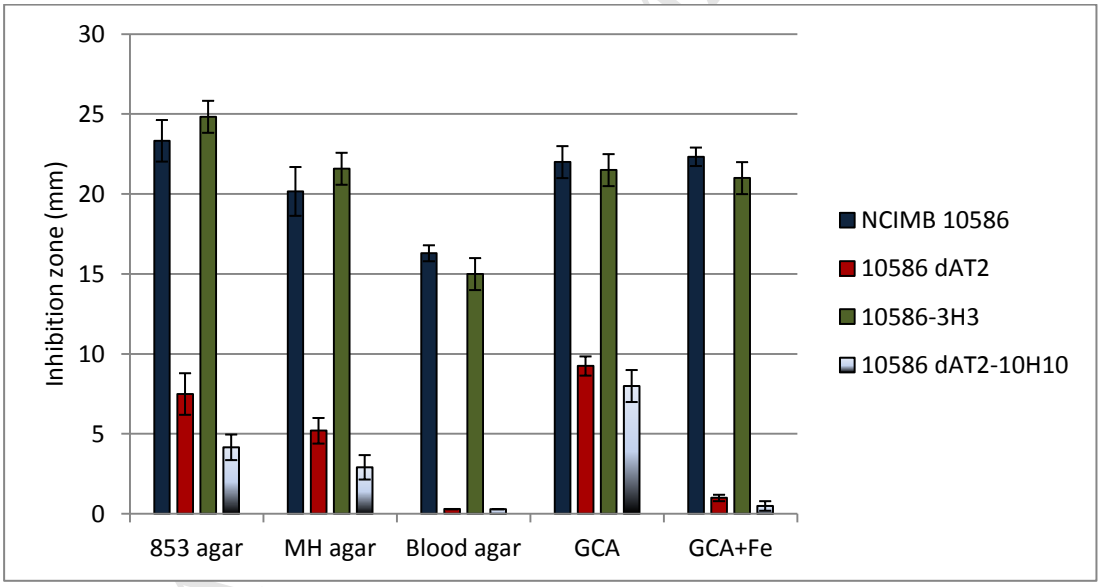
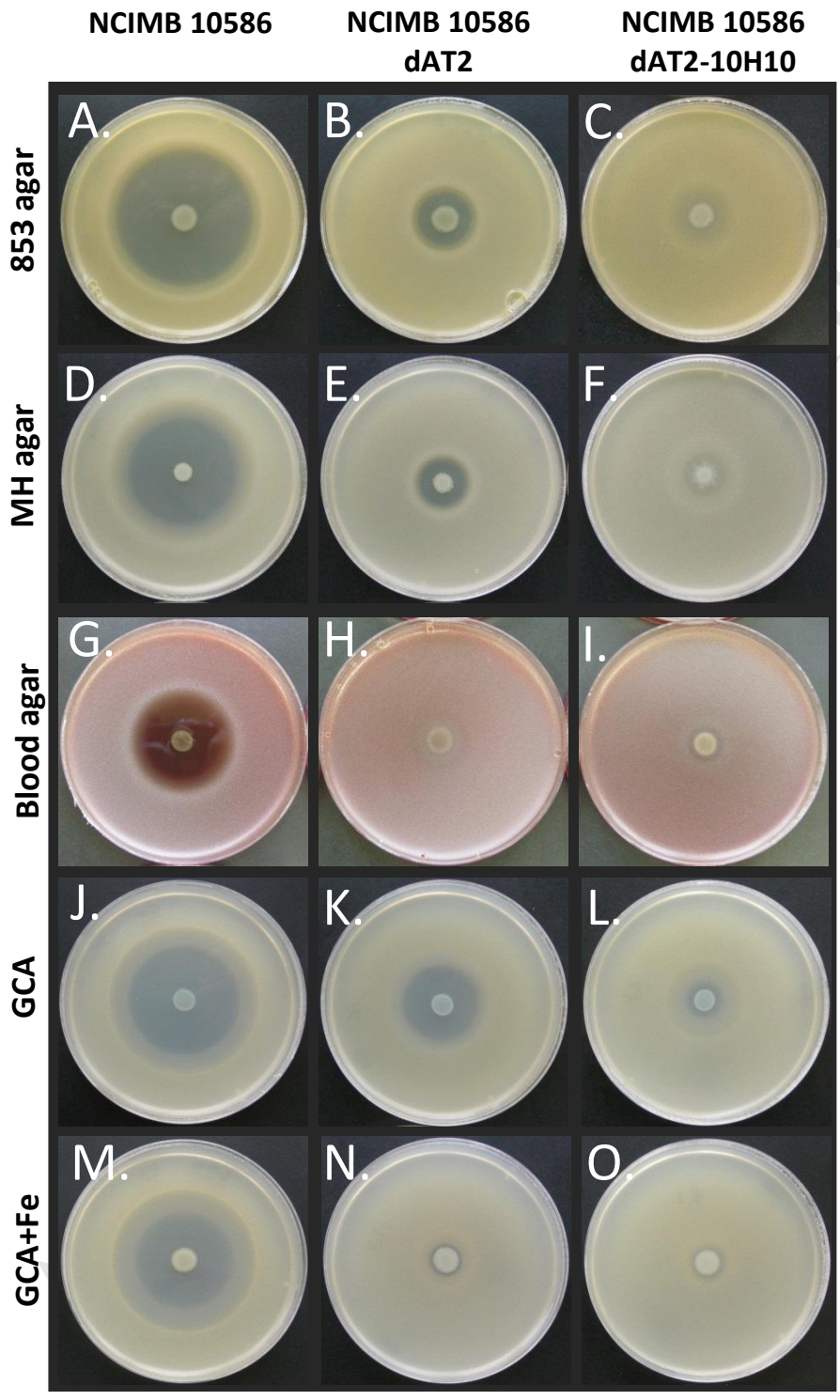


Fig. 4. Comparison of the *in vitro* antagonism of *P. fluorescens* NCIMB 10586 (first column), the mupirocin-negative mutant 10586ΔAT2 (second column) and the double mupirocin/pyoverdine-negative mutant 10586ΔAT2-10H10 (last column) against *S. aureus* 383 on 853 agar (A-C), MH agar (D-F), blood agar (G-I), GCA (J-L) and GCA+Fe medium (M-O).

Fig. 4.


NCIMB 10586
NCIMB 10586 dAT2
NCIMB 10586 dAT2-10H10
853 agar
MH agar
Blood agar
GCA
GCA+Fe
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.
M.
N.
O.

Supplementary Table S1. List of *Pseudomonas* type strains obtained from ATCC (American Type Culture Collection, US), BCCM/LMG Culture Collection (Belgium), CCUG (Culture Collection, University of Göteborg, Sweden), CFBP (French Collection of Plant associated bacteria, France), CFML (Collection de la Faculté de Médecine de Lille, France), CIP (Collection de l'Institut Pasteur, France) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

Species	Strain	Biological origin	Geographic origin
P. abietaniphila	CIP 106708^{T}	Aerated lagoon of bleached kraft pulp mill effluent	Kamploops British Columbia Canada
P. aeruginosa	ATCC 10145^{T}		
P. agarici	CFBP 2063^{T}	Mushroom (*Agaricus bisporus*)	New Zealand
P. alcaliphila	CCUG 136^{T}	Seawater	Near the coast of Rumoi, Hokkaido, Japan
P. amygdali	DSM 7298^{T}	Almond, *Prunus dulcis*	Greece
P. anguilliseptica	CCUG 35503^{T}	Pond-cultured eels (*Anguilla japonica*)	Japan
P. antarctica	CIP 108466^{T}	Cyanobacterial mat	Pond L3 Wright Valley Adam's glacier stream 1 Antarctica
P. argentinensis	CIP 108775^{T}	*Chloris ciliata*, rhizopshere	Cordoba, Argentina
P. arsenicoxydans	CCUG 58201^{T}	Sediment	Chile, Atacama Desert, Camarones Valley
P. asplenii	ATCC 23835^{T}	*Asplenium nidus*	
P. avellanae	CIP 105176^{T}	Hazelnut (*Corylus avellanea*)	Greece
P. azotifigens	DSM 17556^{T}	Compost pile	Japan:Okinawa, Nakijin
P. azotoformans	DSM 18862^{T}	Paddies	Japan
P. baetica	LMG 25716^{T}	Diseased *Dicologoglossa cuneata*	
P. balearica	DSM 6083^{T}	Wastewater treatment plant	Mallorca, Spain
P. bauzanensis	LMG 26048^{T}	Soil contaminated with hydrocarbon and heavy metal from an industrial site	Italy, South Tyrol, Bozen
P. benzenivorans	DSM 8628^{T}	Soil and groundwater contaminated with chlorobenzene	USA, Gulf Coast

P. brassicacearum subsp. *brassicacearum*	DBK11^{T}	Rhizoplane of *Brassica napus*	Dieulouard, France
P. brassicacearum subsp. *neoaurantiaca*	CIP 109457^{T}	Rhizophere of hemp plants	Ukraine, near Kiev
P. brenneri	DSM 15294^{T}	French natural mineral water after bottling	Lille, France
P. caricapapayae	CFBP 3204^{T}	*Carica papaya*	Brazil
P. cedrina subsp. *cedrina*	LMG 23661^{T}	Spring water	Lebanon
P. cedrina subsp. *fulgida*	DSM 14938^{T}	Phyllosphere of grasses	Paulinenaue, Germany
P. chlororaphis subsp. *aurantiaca*	ATCC 33663^{T}		
P. chlororaphis subsp. *aureofaciens*	LMG 1245^{T}	Maas River clay suspended in kerosene for three weeks	Netherlands
P. chlororaphis subsp. *chlororaphis*	ATCC 9446^{T}	Plate contaminant	
P. cichorii	CFBP 2101^{T}	*Cichorium endivia*	Germany
P. citronellolis	CFBP 5585^{T}	Soil under pine trees	
P. composti	DSM 25648^{T}	Mixed vegetables and animal waste compost	
P. congelans	CFBP 7019^{T}	Phyllosphere of grasses	Germany
P. costantinii	CFBP 5705^{T}	*Agaricus bisporus*	Finland
P. corrugata	LMG 2172^{T}	*Lycopersicon esculentum* (tomato) pith necrosis	United Kingdom
P. cremoricolorata	DSM 17059^{T}	*Oryza sativa*	Japan
P. cuatrocienegasensis	LMG 24676^{T}	Water (evaporating lagoon)	Cuatro Ciénegas, Coahuila state, Mexico
P. deceptionensis	LMG 25555^{T}	Marine sediment	Deception Island, South Shetland Islands, Antarctica
P. delhiensis	DSM 18900^{T}	Soil sample contaminated by polycylic aromatic compounds of fly ash dumping site	India
P. duriflava	DSM 21419^{T}	Desert soil	Xinjiang Province, China
P. entomophila	L48^{T}	*Drosophila*	Calvaire, Guadeloupe, France
P. extremaustralis	DSM 17835^{T}	Temporary water pond	Danco Coast, Cierva Point, Antarctica
P. extremorientalis	CCUG 51517^{T}	Drinking water reservoir	Vladivostock City, Russia
P. ficuserectae	CCUG 32779^{T}	*Ficus erectae*	

P. flavescens	CFBP 5586^{T}	Canker tissue of *Juglans regia*	California
P. fluorescens	ATCC 13525^{T}	Pre-filter tanks	UK
P. fragi	ATCC 4973^{T}		
P. fulva	DSM 17717^{T}	Rice paddy	Japan
P. fuscovaginae	CFBP 2801^{T}	*Oriza sativa*	
P. gessardii	CFML 95-251^{T}	Mineral water	France
P. graminis	CCUG 51504^{T}	Surface plant material from a mixed meadow	Germany
P. grimontii	DSM 17515^{T}	Mineral water	France
P. guariconensis	LMG 27394^{T}		
P. guineae	CFBP 7180^{T}	Soil	Deception Island (South Shetland Islands), Antarctica
P. helmanticensis	LMG 28168^{T}	Forest soil	Salamanca, Spain
P. japonica	CCUG 59367^{T}	Activated sludge from sewage treatment plant	Adachi-ku, Odaii, Tokyo, Japan
P. jessenii	CFML 95-307^{T}	Mineral water	France
P. jinjuensis	DSM 16612^{T}	Agricultural soil	Korea
P. kilonensis	DSM 13647^{T}	Agricultural soil	Germany
P. knackmussii	DSM 6978^{T}	Sewage plant	Germany
P. koreensis	CCUG 51519^{T}	Agricultural soil	Korea
P. kuykendallii	LMG 26364^{T}	Bioreactor that degrade the herbicide hexazinone, inoculated with soil	Wiggins, Colorado, USA
P. libanensis	CFML 96-195T^{T}	Spring water	Lebanon
P. lini	D-LE411J^{T}	Bulk and rhizospheric soil	Dijon, France
P. lundensis	DSM 6252^{T}	Prepacked beef	Sweden
P. lurida	CCUG 54630^{T}	Phyllosphere of grasses	Germany
P. lutea	DSM 17257^{T}	Soil	Spain
P. luteola	CIP 102995^{T}	Human wound	
P. mandelii	CFML 95-303^{T}	Mineral water	France
P. marginalis	ATCC 10844^{T}	*Cichorium intybus* imported from Belgium	USA
P. marincola	LMG 24752^{T}	Deep-sea brittle star	Fiji, at a depth of 480 m
P. mediterranea	CFBP 5447^{T}	Pith necrosis on tomato plant	Italy
P. mendocina	ATCC 25411^{T}	Soil, enrichment with ethanol	

P. mohnii	CCUG 53115^{T}	Sequencing batch reactor treating paper mill effluent, enrichment with isopimaric acid	Canada
P. monteilii	DSM 14164^{T}	Human bronchial aspirate	France
P. moorei	DSM 12647^{T}	Soil samples	
P. moraviensis	DSM 16007^{T}	Soil besides highway	Czech Republic
P. mosselii	ATCC BAA-99^{T}	Medical specimen	France
P. mucidolens	CCUG 1424^{T}	Musty egg	
P. nitroreducens	ATCC 33634^{T}	Oil-brine	Japan
P. oleovorans subsp. *oleovorans*	CFBP 5589^{T}	Cutting fluid	
P. oleovorans subsp. *lubricantis*	DSM 21016^{T}	Contaminated metalworking fluids	Houghton, Michigan, USA
P. orientalis	DSM 17489^{T}	Spring water	Lebanon
P. otitidis	DSM 17224^{T}	Ear of patient with acute Otitis externa	USA
P. palleroniana	CFBP 4389^{T}	*Oryza sativa*	Cameroon
P. panacis	DSM 18529^{T}	Rusty root lesions of Korean ginseng	South Korea
P. panipatensis	LMG 24738^{T}	Oil contaminated soil	Panipat Oil Refinery, India
P. parafulva	DSM 117004^{T}	*Oryza sativa*	Japan
P. peli	CIP 109374^{T}	Antarctic green alga *Pyramimonas gelidicola*	Antarctica
P. plecoglossicida	CIP 106493^{T}	Diseased ayu, *Plecoglossus altevelis*	Japan
P. poae	CFBP 6764^{T}	Grasses, phyllopshere	Paulinenaue, Brandenburg, Germany
P. pohangensis	DSM 17875^{T}	Sea shore sand	Korea
P. prosekii	LMG 26867^{T}	Rock biofilm	James Ross Island, Waterfall, west side of Lachmen Crags Antarctica
P. protegens	CHA0^{T}	Soil suppressing black root rot of tobacco (*Nicotiana glutinosa*)	Switzerland
P. proteolytica	CIP 108464^{T}	Cyanobacterial mat samples	Antarctica
P. putida	CFBP 2066^{T}	Soil, lactate enrichment	United States
P. reinekei	CCUG 53116^{T}	Aerobic zone of Elbe sediment enriched with 4-chlorosalicylate	Germany
P. resinovorans	CCUG 2473^{T}	Soil	France, Vienne

P. rhodesiae	ATCC 17764^{T}	Natural mineral water	France
P. sabulinigri	DSM 23971^{T}	Black sand originating from black volcanic basalt rock	Jeju Island, Soesgoggak, Korea
P. salomonii	CFBP 2022^{T}	*Allium sativum*	France
P. saponiphila	DSM 9751^{T}		Michigan, USA
P. savastanoi	CIP 103721^{T}	*Olea europaea*	Yugoslavia
P. segetis	CCUG 54777^{T}	Soil	Korea
P. simiae	DSM 18861^{T}	Lung of female monkey (*Callithrix geoffroyi*) with pneumonitis and pneumonia	Spain
P. straminea	CCUG 12539^{T}	Japanese unhulled rice	Japan
P. stutzeri	ATCC 17588^{T}	Spinal fluid	
P. synxantha	DSM 18928^{T}	Cream	Iowa, USA
P. syringae subsp. *syringae*	ATCC 19310^{T}	*Syringa vulgaris*	United Kingdom
P. taetrolens	CCUG 560^{T}	Musty egg	
P. taiwanensis	DSM 21245^{T}	Soil	Tamkang University, Taipei, Taiwan
P. thivervalensis	CFBP 5754^{T}	Rhizoplane of *Brassica napus*	Sexy-les-Bois, France
P. tolaasii	CFBP 2068^{T}	*Agaricus bisporus*	United Kingdom
P. trivialis	CFBP 6765^{T}	Grasses, phyllopshere	Paulinenaue, Brandenburg, Germany
P. umsongensis	CIP 108618^{T}	Agricultural soil	Korea
P. vancouverensis	ATCC 700688^{T}	Forest soil	Canada
P. veronii	CFML 92-134^{T}	Mineral water	France
P. viridiflava	ATCC 13223^{T}	Dwarf or runner bean	Switzerland
P. vranovensis	DSM 16006^{T}	Soil besides highway	Czech Republic
P. xiamenensis	DSM 22326^{T}	Activated sludge sample collected at Qianpu sewage treatment plant	Fujian, Xiamen, China

Supplementary Table S2. List of *Pseudomonas* non type strains obtained from ATCC (American Type Culture Collection, US), BCCM/LMG Culture Collection (Belgium), CFBP (French Collection of Plant associated bacteria, France), CFML (Collection de la Faculté de Médecine de Lille, France), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany).

Strain	Species	Biological origin	Geographic origin
ATCC 17400	*P. fluorescens*	Egg yolk	
ATCC 39167	*P. putida*		
ATCC 43928	*P. chlororaphis*	Agricultural field	Denmark
CFBP 2123	*P. fluorescens*	Tap water	Netherlands
CFBP 2124	*P. fluorescens*	Lactate-enriched water	
CFBP 2125	*P. fluorescens*	Enrichment with naphthalene	
CFBP 2129	*P. fluorescens*	Hydrocarbon enrichment	Berkeley, United States
CFBP 2131	*P. fluorescens*	Soil	
CFBP 2392		Rhizosphere of *Phaseolus vulgaris*	France
CFBP 2461	*P. putida*	Soil	France
CFBP 3140	*P. putida*		
CFBP 3142	*P. putida*	Soil	
CFBP 3143	*P. putida*	Soil	
CFBP 3150	*P. fluorescens*	Soil	
CFBP 3153	*P. marginalis pv. marginalis*	Pleural fluid	
CFBP 3155	*P. chlororaphis*	Farm soil	Peoria, USA
CFBP 4628	*P. putida*	*Lycopersicon esculentum*	Maine et Loire Angers, France
CFBP 4966	*P. putida*	*Lycopersicon esculentum*	Guadeloupe, France
CFBP 4967	*P. fluorescens*	*Lycopersicon esculentum*	Guadeloupe, France
CFBP 4970	*P. putida*	*Lycopersicon esculentum*	Guadeloupe, France
CFBP 4971	*P. putida*	*Lycopersicon esculentum*	Guadeloupe, France
CFBP 4976	*Pseudomonas* sp.	Tabac	Maine et Loire Angers, France
CFBP 5891	*P. putida*	*Zea mays*	Maine et Loire, France
CFBP 5898	*P. putida*	*Zea mays*	Maine et Loire, France
CFBP 5913	*P. putida*	*Zea mays*	Maine et Loire, France

CFBP 5921	*P. putida*	*Lycopersicon esculentum*	Guadeloupe, France
CFBP 5930	*P. putida*	*Lycopersicon esculentum*	Angers, France
CFBP 5933	*P. putida*	*Lycopersicon esculentum*	Angers, France
CFML 90-33	*P. putida*		
CFML 90-40	*P. putida*		
CFML 90-42	*P. putida*		
CFML 90-44	*P. putida*		
CFML 90-51	*P. putida*	Clinical	
CFML 90-136	*P. putida*		
CFML 95-275	*Pseudomonas* sp.		
CFML 96-132	*Pseudomonas* sp.		
CFML 96-188	*Pseudomonas* sp.	Spring water (Kadicha)	Lebanon
CFML 96-312	*Pseudomonas* sp.		
CFML 96-318	*Pseudomonas* sp.		
CFML 96-319	*Pseudomonas* sp.		
DSM 3602	*P. stutzeri*	Black earth soil	Gatton, Queensland, Australia
DSM 50106	*P. fluorescens*	Seawater	
LMG 1244	*P. fluorescens*	Polluted seawater	Dresund, Denmark
LMG 14576	*P. fluorescens*	Well water	
LMG 14677	*P. fluorescens*		
LMG 2189	*P. fluorescens*		
LMG 5831	*P. fluorescens*	Polluted natural stream	South Copenhagen, Denmark
LMG 5848	*P. fluorescens*	*Cichorium intybus*, leaves showing blackening of vascular system	United Kingdom

Supplementary Table S3. List of *Pseudomonas* strains and isolates obtained from different laboratories and their biological and geographic origin.

***Pseudomonas* strains and isolates**	**Biological and geographic origin**	**Reference**
LUBF-l55, LUBF-l59, LUBF-l69, LUBF-l88, LUBF-l102, LUBF-l104, LUBF-l107, LUBF-l112, LUBF-l212, LUBF-l410, LUBF-l615, LUBF-l715	Spoilage of *Sparus aurata* stored under various conditions	[1]
SWPA 6, SWPA 40A, SWPA 40B, SWPA 36A, SWPA 36B, SWPA 36C, SWPA 10.20A, SWPA 10.20B, SWPA 10.20C, 115 thio, 120, LS2-01, 545, 415, 250A, 250B, 250C, 250D, SWPA0004-10, SW4, SW5, SW6, SW7, SW33, SW11, SW16, SW29, SWPA 115 25-F, SWPA 820 18-F, SWPA 2404 25-T	Seawater, North Sea (few km before the coast, 15m depth)	Jean-Paul Pirnay, Belgium (unpublished)
TKM iso1, TKM iso2, TKC, LKA, TKD1, 4T, 5TWPS, 3TWPS, TW1, 2T	Lake Tanganyika	Jean-Paul Pirnay, Belgium (unpublished)
Br232, Br252	Unfiltered tap water, Burn Centre, Queen Astrid Military Hospital, Brussels, Belgium	Jean-Paul Pirnay, Belgium (unpublished)
Br996	Water tank of a ship	Jean-Paul Pirnay, Belgium (unpublished)
Mex8A, Mex8B1, Mex8C1, Mex8D1	Water of a cenote, Loltun, Mexico	Jean-Paul Pirnay, Belgium (unpublished)
K-SR5, K-SR6, K-SR7, K-SR8, K-SR9, K-SR10, K-SR11, K-SR21, K-SR33	Rhizosphere *Salsola vermiculata*, Bèchar, Algeria	Khadidja Chafi, Algeria (unpublished)
MFY0	Raw milk	[2]
MFY30, MFY31, MFY32, MFY33, MFY52, MFY76, MFY80,	Bulk soil	[2]

MFY81, MFY122, MFY140, MFY152, MFY160, MFY138, MFY143, MFY146, MFY220, MFY338, MFY245		
OE28.3	Rhizosphere	[2]
MFY59, MFY63,	Urine, Pitié Salpêtrière Hospital, France	[2]
MFY57, MFY68, MFY163	Blood, Pitié Salpêtrière Hospital, France	[2]
MFY161	Blood/urine, Charles Nicolle Hospital, France	[2]
MFY78, MFY79	Dun River, France	[2]
MFY70	Suppuration, Pitié Salpêtrière Hospital, France	[2]
MFY65	Infected sinus	[2]
MFY61	Articular infection	[2]
MFY162	Sputtum, Charles Nicolle Hospital, France	[2]
MFY69	Gastric fluid, Pitié Salpêtrière Hospital, France	[2]
MFY71	Clinical	[2]
R2f, B13, B125, B136, B163, B215, B329	Rhizosphere	Josselin Bodilis, France (unpublished)
R-35697, R-35701, R-35703, R-35705, R-35706, R-35709, R-35710, R-35717, R-35719, R-35702, R-35711, R-35721, R-35723, R-35724, R-35708, R-35700, R-35720, R-35707, R-35725	Milk, Belgium	[3]

PD4, PD5, PD11, PD13, PD15, PD22, PD28, PD31	Arable soil used for potato production, New Brunswick, Canada	[4]
P. putida BTP1, *P. fluorescens* BTP2, BTP7', BTP9, BTP14, PP, *P. putida* PutC		Collection obtained from Marc Ongena, Belgium
P. putida Irc204	Soil, Belgium	IRMW
P. fluorescens E211, *P. kilonensis*/*brassicacearum* G11, *P. fluorescens* G27	Rhizosphere of *Echinochloa crus-galli*/*Galium mollugo*	[5]
P. putida WCS358, WCS365, *P. fluorescens* WCS374		
Pseudomonas sp. B10, *P. fluorescens* G153, *P. fluorescens* G166, *P. fluorescens* G173, *P. chlororaphis* D-TR133, *P. putida* G4R, *P. fluorescens* 99-13, *P. fluorescens* PL7, *P. fluorescens* PL8, *P. fluorescens* PL9, *Pseudomonas* sp. D46, *Pseudomonas* sp. D47, *P. fluorescens* Pflii, *P. fluorescens* Pfl12, *P. fluorescens* PflW, *Pseudomonas* sp. F360, *P. fluorescens* 9AW, *P. fluorescens* 1547, SB8.3, *Pseudomonas* sp. 2908, *P. fluorescens* 1.3, *P. fluorescens* 18.1, HR6, *P. monteilii* Lille 1, *P. fluorescens* 51W, *P. rhodesiae* Lille 25, Gwose, *Pseudomonas* sp. A214, *P. fluorescens* A225, *P. putida* Thai, *P. aeruginosa* R', *Pseudomonas* sp. 6-10, LBSA1, IB3, 7SR1, ML35, Malar, *P. aeruginosa* Pa6, Lille 17, *Pseudomonas* sp., *Pseudomonas* sp. F317, *P. fuscovaginae* G17, *Pseudomonas* sp. G400		Collection obtained from Jean-Marie Meyer, France
P. putida GM 10090 (G297), *P. putida* GM 11799.1 (G309), *P. putida* GM 12220 (G314)	Plant rhizosphere	[6]
P. putida GS4 (GS12059), *P. putida* GS35 (GS12082), *P. putida* GS37 (GS12064)	Plant rhizosphere (banana tree)	[7]

SR11, SR14, SR18, SR28, SR44, SR45, SR54, SR56, SR64, SR66, SR67, SR68, SR70	Metal-contaminated sediment	[8]
P. brassicacearum R1-4	Rhizosphere oilseed rape	[9]
P. thivervalensis DR5, *P. brassicacearum* BGCR2-9(1)	Bacterial endophyte from field-grown *Solanum nigrum*	[10]
AF76	Rhizosphere ground nut (*Arachis hypogaea* L.), India	[11]

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Supplementary Table S4. Primers and their sequences used to amplify part of the tailoring genes of the mupirocin gene cluster.

Primer	Sequence 5'-3'	Size amplicon	Primer	Sequence 5'-3'	Size amplicon
mupZ-F	ATGAATCGCACCTGCATGGC		mupL-F	ATGCAACTGATCACGCACGA	
mupZ-R	GAACTGCGGCITCATAAACG	324 bp	mupL-R	ATCCACGTCTGCCTCACCCT	936 bp
TEB-F	TGACGCTGCTCAATCACGCT		mupM-F	ATGAGTACGGAAGGAAGTGG	
TEB-R	ACCTGCITGAGGGACACCAC	440 bp	mupM-R	GCGGATGAACCAGGACTCTA	1221 bp
mmpC-F	GGGTCGCAATACCGTCACAT		mupO-F	GACATCGTGGGAAAGAGAGG	
mmpC-R	CGTTGAGCGATTTCGACAAT	2140 bp	mupO-R	GTAGGAGATGATGGCGTGGIT	775 bp
MetD3-F	ATGITCCCCGAGGGTTCCAT		mupP-F	GTGAGCAGGTCCGAACTTG	
MetD3-R	GCTCAGCTCGITCAACAACA	470 bp	mupP-R	TCGGTAAAGGGCAITGAAGTG	699 bp
mupC-F	AAGGTCGTCGGGITCACTGG		mupQ-F	ATGAGAGAGGAACGTAATTGG	
mupC-R	CGGTAAGTGGCTTCGGACAA	531 bp	mupQ-R	CCTTGAAATCITCCAACCCT	1279 bp
macpA-F	ATGAACCCTGAAAGGCGGAA		mupS-F	ATGACTGATGCAGTTTCTGACG	
macpA-R	GITGACGGCACTCATCCAIT	222 bp	mupS-R	TGCGGGTCAAGCCATCC	535 bp
mupD-F	ATGCGTAGGCAGGTAGTCGT		mupT-F	ITGGTAGAGCATCCGCCC	
mupD-R	GGTGAGATGITGCCCGITGA	708 bp	mupT-R	TCACGTACCGCACTCCAGCC	351 bp
mupE-F	GGCGCTTCAACTGGTTGATA		mupU-F	ATGGAACAGITGACCCCTGG	
mupE-R	CCGAAAGAGCCATGAATGTG	864 bp	mupU-R	GCGACACTACCGCCACIT	1336 bp
mupF-F	TCAGGGTGCTGTCGGGGATA		mupV-F	AAAGCTCTATGTGACCGGCG	
mupF-R	GGCAGCCATATTGTGGAAAA	969 bp	mupV-R	GCTGGAITGGCGGCTCT	1630 bp
mupG-F	ATGGCAAGCTCTGACACGCA		mupW-F	GCAAATTGATCGAGCACGTC	
mupG-R	ACTGCCGTGCGGGTTGATAT	918 bp	mupW-R	GGCGTGCTCATAGGTGTCG	1208 bp
mupH-F	ATGACGAGGCAAGTGGGTAT		mupX-F	TGGTCAGCGCGTTCCACTTT	
mupH-R	CCCAGCAGGAITGACTCGTA	1121 bp	mupX-R	AAGAGTGAGGCGGGCTACG	1510 bp
mupJ-F	ATGAACITCCAGGCCACCGA		mupl-F	TAGCATTGACTGCGTCCAGG	
mupJ-R	TGGCTGACATAGCGGTGGAT	740 bp	mupl-R	GAACATATCACCACGITATCTGGA	555 bp
mupK-F	GGACAGTGTCATCGATITTCAGG		mupR-F	TGCITGAAGACAITCTGATG	
mupK-R	CCAACTCCATCGCCAACTCC	581 bp	mupR-R	GCCACCCACITCAGACA	550 bp

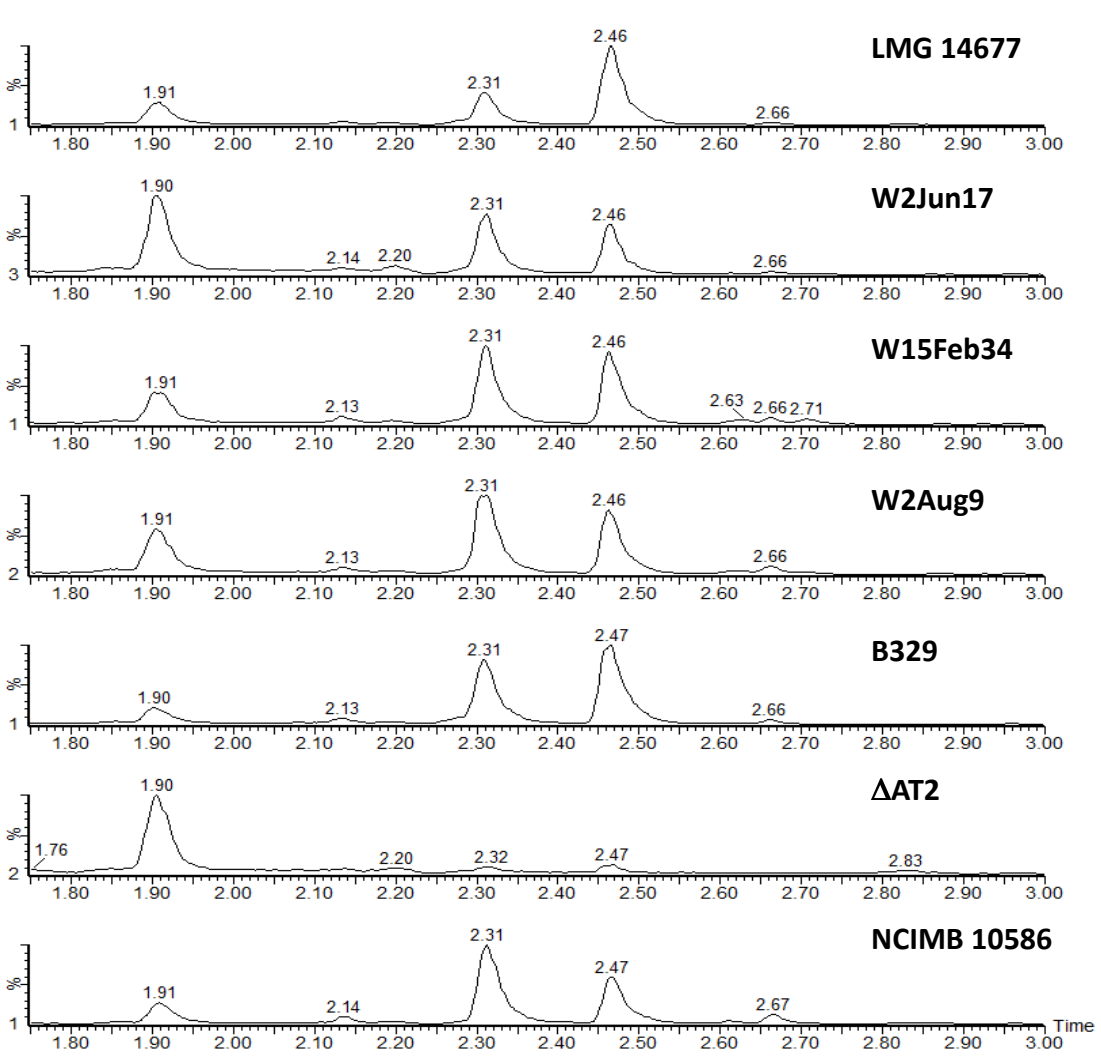
Supplementary Table S5. The interspecies similarity values for the *rpoB* gene of the mupirocin producing strains.

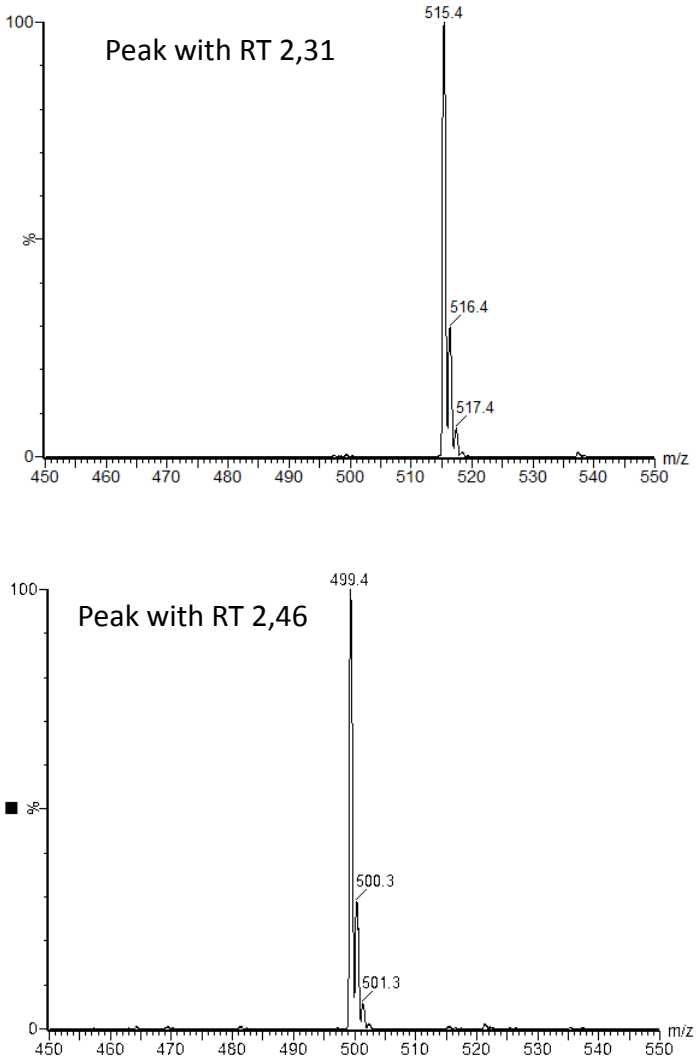
Species	**1**	**2**	**3**	**4**	**5**	**6**
1. *P. fluorescens* NCIMB 10586	100.0					
2. *Pseudomonas* sp. W2Aug9	99.13	100.0				
3. *Pseudomonas* sp. W2Jun17	99.02	99.89	100.0			
4. *Pseudomonas* sp. W15Feb34	99.02	99.67	99.57	100.0		
5. *Pseudomonas* sp. B329	99.57	99.35	99.24	99.24	100.0	
6. *P. fluorescens* LMG 14677	99.89	99.89	99.79	99.79	99.24	100.0

Supplementary Fig. S1. Part of the *mmpD* gene of *P. fluorescens* NCIMB 10586 that aligns with the complementary sequence of the PltBf and PltBr primers. The nucleotides that mismatch are indicated in red. There was only 1 internal nucleotide mismatch with the forward primer and 3 mismatches of 4 nucleotides (thereby discarding the nucleotide at the 5'end) with the reverse primer.

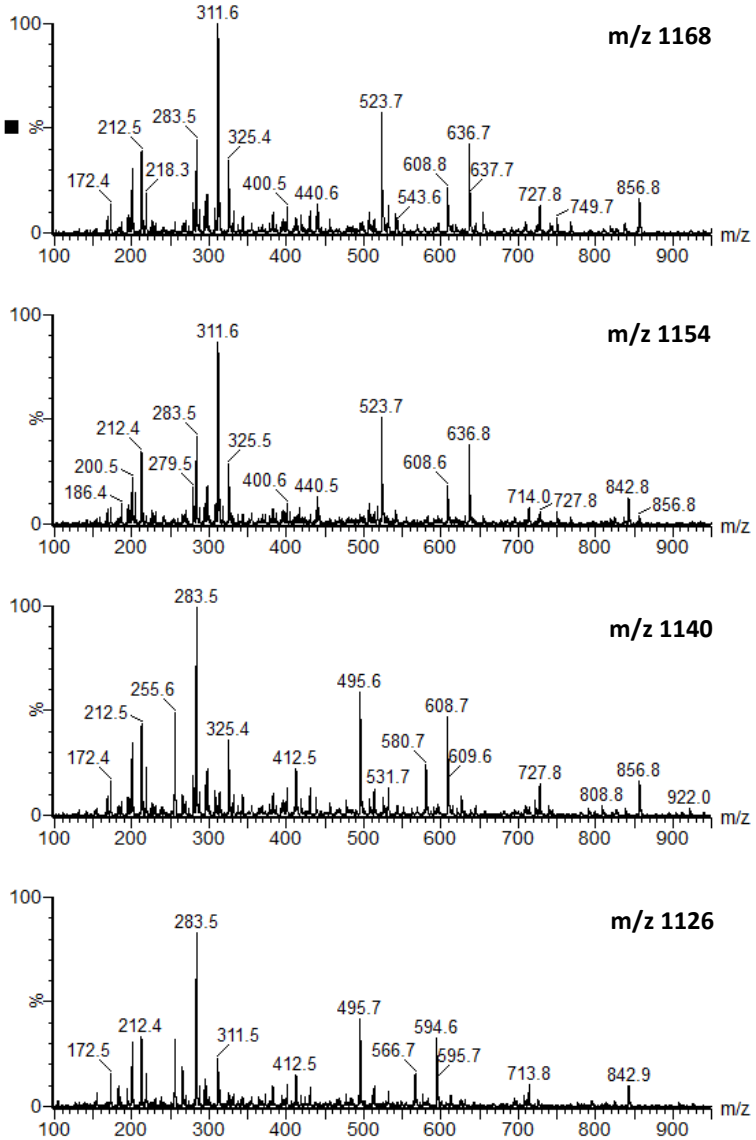
```
mmpD NCIMB 10586:       5′-CGGATCATGGACCCCCAGC-3′
                           ||||  ||||||||||||||
PltBf primer:           5′-CGGAGCATGGACCCCCAGC-3′

mmpD NCIMB 10586:       5′-ATGGCCGATATTGGATTTGGCCGAG-3′
                            ||  ||||||||||   |||  |||||
PltBr primer:           5′-GTGCCCGATATTGGTCTTGACCGAG-3′
```

Supplementary Fig. S2. Identification of main peaks in supernatants of the reference strain *P. fluorescens* NCIMB 10586, the mupirocin-negative mutant ΔAT2 and the mupirocin producing strains *P. fluorescens* LMG 14677 and *Pseudomonas* sp. W2Jun17, W15Feb34, W2Aug9 and B329. The peak with retention time (RT) 2.31 is pseudomonic acid B, the peak with RT 2.46 is pseudomonic acid A.





Supplementary Fig. S3. Fragmentation patterns obtained for the main lipopeptide molecular ions in *Pseudomonas* sp. W2Aug9.



Representative ions (m/z)	Fragment species
283,6	C10FA-Leu
412,6	C10FA-Leu-Glu
495,6 (-H_2O); 513,6 (-2H_2O)	C10FA-Leu-Glu-Thr
594,6 (-H_2O); 566,6 (-2H_2O)	C10FA-Leu-Glu-Thr-Val
608,6 (-H_2O); 580,6 (-2H_2O)	C10FA-Leu-Glu-Thr-Ile/Leu
186 (204, +H_2O)	Ser-Val
200 (218, +H_2O)	Ser-Ile/Leu
299 (313, +H_2O)	Leu-Ser-Val
313 (331, +H_2O)	Leu-Ser-Ile/Leu
386 (404, +H_2O)	Ser-Leu-Ser-Val
400 (418, +H_2O)	Ser-Leu-Ser-Ile/Leu
499 (517, +H_2O)	Leu-Ser-Leu-Ser-Val
513 (531, +H_2O)	Leu-Ser-Leu-Ser-Ile/Leu
612 (630, +H_2O)	Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu
695 (713, +H_2O)	Thr-Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu
824 (842, +H_2O)	Glu-Thr-Val/Ile/Leu-Leu-Ser-Leu-Ser-Val/Ile/Leu

Ion assignement:

m/z 1126 peak 1:C_{10}FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L
m/z 1126 peak 2: C_{10}FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin
m/z 1140 peak 1:C_{10}FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D
m/z 1154 peak 1: C_{12}FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Val; Massetolide L
m/z 1154 peak 2: C_{12}FA-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile/leu; Massetolide F or Viscosin
m/z 1168 peak 1: C_{12}FA-Leu-Glu-Thr-Ile/Leu-Leu-Ser-Leu-Ser-Ile/Leu; Massetolide A or D